Muscarinic receptor activation of AMP-activated protein kinase inhibits orexigenic neuropeptide mRNA expression
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Running Title: AMPK regulates neuropeptide mRNA expression
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AMP-activated protein kinase (AMPK) plays a crucial role in both cellular and whole body energy homeostasis. Here we demonstrate that the muscarinic receptor agonist carbachol activates AMPKα1-containing complexes in the human SH-SY5Y cell line via a mechanism specific for the AMPK upstream kinase, CaMKKβ. Activation of AMPK inhibits mRNA expression of the orexigenic neuropeptides AgRP and MCH but surprisingly has no effect on NPY mRNA, a neuropeptide previously shown to be regulated by AMPK. Rather than restoring mRNA levels to baseline, pharmacological inhibition of CaMKKβ or AMPK greatly increases AgRP and MCH mRNA expression. These data support a hypothesis that modulating basal AMPK activity in the hypothalamus is essential for maintaining tight regulation of pathways contributing to food intake.

AMPK is a key metabolic enzyme in the regulation of energy homeostasis (1). At a cellular level, AMPK is activated in response to diverse physiological and pathological stimuli that cause ATP depletion. Subsequently, AMPK acts to maintain the AMP:ATP ratio through inhibition of pathways that catabolise ATP and promotion of ATP-generating pathways (2).

AMPK is a heterotrimeric serine/threonine kinase, comprising a catalytic α subunit and regulatory β and γ subunits. There are two α, two β and three γ isoforms and all 12 αβγ combinations have been identified in vivo. Activation of AMPK requires the action of upstream kinases that phosphorylate threonine residue 172 within the T-loop activation domain of the α subunit (2). Three AMPK kinases have been identified to date. LKB1 is a tumour suppressor kinase linked to the rare hereditary cancer predisposition, Peutz-Jeghers Syndrome (3). In complex with regulatory proteins STRADα and MO25, LKB1 phosphorylates and activates AMPK (4,5) possibly in response to changes in the AMP:ATP ratio. In addition, Ca²⁺/calmodulin-dependent protein kinase kinase β (CaMKKβ) has also been identified as an AMPK kinase (6-8), phosphorylating and activating AMPK in response to elevated intracellular Ca²⁺ concentrations. Finally TGF-β-activated kinase 1 (TAK1) was very recently implicated in the regulation of AMPK activity in cells and in the heart, although its regulation remains unknown (9,10). Thus, AMPK is well equipped to respond to a diverse range of stimuli.

AMPK is expressed ubiquitously and recent findings have stimulated interest in roles for neuronal AMPK (11). Although prolonged pharmacological activation of AMPK in neuroblastoma cells is reported to induce apoptosis (12), increasing evidence points towards a role for AMPK in neuroprotection. AMPK is activated in response to excitotoxic stimuli in hippocampal neurons and inhibiting AMPK potentiates neurodegeneration (13). More recently, AMPK was shown to interact with and phosphorylate the metabotropic GABA(A) receptor, enhancing the activation of inwardly rectifying K⁺ channels (GIRKs). This mechanism was potentiated by increased intracellular AMP such as might occur during pathological stimuli such as hypoxia or ischaemia and ultimately acts to suppress neuronal excitation, preventing cellular damage (14). In the hypothalamus, AMPK is localised to the arcuate nucleus where it responds to circulating hormones, playing a role in co-ordinating the regulation of appetite and satiety (15,16). On exposure to circulating anorexigenic signals such as leptin, glucose or insulin, AMPK activity is inhibited and conversely, orexigenic signals, such as ghrelin or...
fasting, promote AMPK activity (15-18). Adenoviral injection of constitutively active AMPK into the ventromedial hypothalamus causes increased food intake and body weight and the reverse occurs on injection of dominant negative AMPK (15,16).

In SH-SY5Y human neuroblastoma cells and primary rat acinar cells, AMPK is activated by carbachol, a muscarinic receptor agonist (19,20). In SH-SY5Y cells, the predominant muscarinic receptor has been pharmacologically determined as the M3 subtype (21). Similarly to AMPK, M3 pathways have also been implicated in the regulation of food intake. Knockout mice lacking the M3 receptor have reduced food intake and are lean (22) and it was suggested that there is an M3 receptor-dependent pathway downstream of primary appetite-regulating receptors in the hypothalamus. Here we show that in SH-SY5Y cells, exposure to carbachol causes activation of AMPK α1-containing complexes without perturbing the AMP:ATP ratio. We provide evidence that although both LKB1 and CaMKKβ are present and functional in these cells, it is CaMKKβ that mediates the action of carbachol on AMPK. Finally, we show that carbachol and other direct activators or inhibitors of AMPK can significantly modulate the mRNA expression of neuropeptides involved in appetite regulation.

**Experimental Procedures**

**Materials** AMARA peptide substrate, anti-CaMKKβ and anti-AMPK pan-β polyclonal antibodies were produced in house (23). Anti-LKB1 rabbit polyclonal serum antibodies were raised against bacterially expressed full-length mouse LKB1 and characterised in house. Phospho-AMPK (pThr172), AMPKα1, AMPKα2 and phospho-Acetyl CoA Carboxylase rabbit monoclonal antibodies were from Cell Signaling (New England Biolabs, Herts, UK). IRDye Infrared Dye secondary antibodies were purchased from LI-COR (Cambridge, UK). SH-SY5Y human neuroblastoma cell line was from ECACC (Salisbury, UK). Nucleofection reagents were from AMAXA (Cologne, Germany). Recombinant bacterially expressed AMPK (α1β1γ1) was purified as described previously (24). Anti-actin monoclonal antibody, carbachol (carbamoyl chloride), pilocarpine, atropine, retinoic acid, m-3M3FBS and protein A-sepharose were purchased from Sigma (Dorset, UK). TRIZol, tissue culture media and supplements were from Invitrogen (Paisley, UK). STO-609 was purchased from Tocris (Ellisville, US). U73122, Compound C and 5-aminimidazole-4-carboxamide ribonucleoside (AICAR) were obtained from Calbiochem (Nottingham, UK). AMPK activator A-769662 was purchased from the University of Dundee (Dundee, UK). Quantitect SYBR green One Step RT-PCR kit, Quantitect primer assay kits (for human 18S, POMC, NPY, MCH and AgRP sequences) and RNeasy kit were purchased from Qiagen (West Sussex, UK).

**Preparation of SH-SY5Y cell lysates and RNA.** SH-SY5Y cells were maintained in a 1:1 mix of DMEM:F12 media supplemented with 2mM glutamine and 10% foetal bovine serum according to ECACC guidelines. For differentiation, cells were transferred into media containing 1% foetal bovine serum and 10 mM retinoic acid for 4-6 days. Cells were treated with 0.5 mM hydrogen peroxide (15 min), pilocarpine (10µM, 30 min) or carbachol (for times and concentrations indicated in Fig. 1). When required, cells were pretreated with STO609 (10µg/ml media) or Compound C (10µM) for 1hr or atropine (10µM) for 30 min before AMPK activation by carbachol. Direct AMPK activation was achieved by treatment with A-769662 (200µM, 1hr) or AICAR (2mM, 30min). For preparation of whole cell lysates, cells were rinsed briefly in phosphate-buffered saline (PBS) before lysis in ice-cold HEPES Buffer A containing Triton (50 mM HEPES pH 7.5, 50 mM NaF, 5 mM sodium pyrophosphate, 1 mM EDTA, 1 mM dithiothreitol, 0.1 mM phenylmethylsulphonyl fluoride, 157 µg/ml benzamidine, 10% [v/v] glycerol, 1% [v/v] Triton X-100). Insoluble material was removed by brief centrifugation and the supernatant fraction used for subsequent analysis. For preparation of total RNA, SH-SY5Y cells were harvested in TRIZol (1ml per 1 x 10⁶ cells) and total RNA extracted in chloroform as per manufacturer’s instructions. The resulting RNA was purified using an RNeasy column (Qiagen) and the manufacturer’s protocol followed.

**siRNA-mediated knockdown of LKB1 and CaMKKβ.** Synthetic RNA duplexes were transfected into SH-SY5Y cells using AMAXA Nucleofection Kit V in accordance with
manufacturer’s protocol. Sequences are as described previously (8,25). Cells were harvested 48h post nucleofection.

**Western Blot Analysis.** Total SH-SY5Y protein lysates (30-50µg) were resolved by 12% SDS-PAGE and transferred to LI-COR-compatible PVDF membrane. Primary antibodies were detected using LI-COR IRDye Infrared Dye secondary antibodies and visualised using an Odyssey Infrared Imager (LI-COR Biotechnology).

**Analyses of intracellular nucleotide levels.** SH-SY5Y cells (1-2x10⁶) were rinsed with phosphate buffered saline and lysed by addition of 5% perchloric acid. Insoluble material was removed by centrifugation (10,000g, 1 min) and nucleotides analysed as described previously (26).

**Imaging of intracellular calcium.** SH-SY5Y cells were plated on poly-L-lysine coated glass coverslips and induced to differentiate, after 24h from plating, by the addition of 10µM retinoic acid. Cells were loaded with the membrane-permeant calcium indicator fluo-4 acetoxymethyl ester (fluo-4-AM, Invitrogen) by incubation in a loading solution of 2.5 µM fluo-4-AM in isotonic physiological saline solution (PSS) supplemented with 0.02% Pluronic F127 (Invitrogen), for 30 minutes at 37°C. Subsequently, cells were incubated for at least 10 minutes in PSS at room temperature to allow for de-esterification. PPS contains 130 mM NaCl, 4.7 KCl, 1mM MgCl₂, 1.2 KH₂PO₄, 1.3 CaCl₂, 20mM Hepes and 5mM glucose, pH 7.4. Coverslips were mounted on a Bioptecs FCS2 imaging perfusion chamber (Bioptecs Inc.) and perfused with PPS. Experiments were conducted at room temperature. Images were acquired using a PerkinElmer Ultraview confocal Live-Cell System (LCS) fitted on a Zeiss AxioVert 200 inverted microscope through a Zeiss Plan-Apochromat 63x 1.4NA oil immersion objective. Fluo-4 was excited with a 488-nm laser line of an Argon Ion laser, and the emitted fluorescence was collected with a 500LP emission filter. Images were collected every 2 s. The average fluorescent signal (F) from a representative area within the cell was analysed along the time, and normalised to the maximal dye fluorescence (Fₘₐₓ) rendering the ratio F/Fₘₐₓ. Fₘₐₓ was measured at the end of the experiment following elevation of intracellular calcium by exposure of the cells to the calcium ionophore ionomycin (10µM) in high Ca²⁺ and high K⁺ solution (30 mM CaCl₂, 125mM KCl, 10mM HEPES pH 7.4).

**AMPK assays.** LKB1, CaMKKβ and AMPK were immunoprecipitated from SH-SY5Y cell lysates (50µg). LKB1 and CaMKKβ reactivation assays were performed for 30 minutes at 37°C in the presence of bacterially expressed recombinant AMPK (5,8). AMPK assays were performed using 0.1mM AMARA peptide substrate as described previously (5,27).

**Analysis of mRNA by quantitative RT-PCR.** Total RNA (10ng) purified from treated or untreated SH-SY5Y cells was subjected to quantitative real time RT-PCR using a Qiagen SYBR green Quantitext protocol and prevalidated Quantitect Primer pairs as per manufacturer’s instructions. Data were normalised to 18S control. Single products were verified by melting curve analysis.

**Statistical analyses.** Data are expressed as mean ± standard deviation from three or more independent experiments. Statistical significance was established by Student’s t-test. Data was considered significant at p<0.05.

### RESULTS

**Carbachol activates AMPKa1 in SH-SY5Y cells.**

Carbachol has been reported to increase AMPK activity (19,20), however the mechanism for activation remains unknown. In order to dissect the pathway, we investigated AMPK activation by carbachol in the SH-SY5Y neuroblastoma cell line. SH-SY5Y cells were treated with varying concentrations of carbachol for between 5 and 30 minutes and endogenous AMPK immunoprecipitated. AMPK activity was increased in a dose- (Fig. 1a) and time-dependent manner (Fig. 1b) with four-fold activation after 5 minutes exposure to 10µM carbachol, subsiding to two-fold at 30 minutes. Carbachol did not activate AMPK in primary cardiomyocytes or hepatocytes (data not shown) suggesting the effect may be relatively specific for neuronal cells. In response to increased AMPK activity, phosphorylation of a
known AMPK substrate, acetyl CoA carboxylase (ACC), was also increased in a time frame correlating with AMPK activation (Fig. 1c). Western blot analysis of SH-SY5Y cell lysates shows that both catalytic α1 and α2 subunit isoforms of AMPK are present although α2 is expressed to a much lesser extent (data not shown). To determine which isoform mediates the response to carbachol, subunit-specific sequential depletion of AMPK was performed by three rounds of immunoprecipitation from the same lysates. The predominant AMPK activity in SH-SY5Y cells was provided by α1-containing complexes (Fig. 1d) and there was a three-fold increase in AMPKα1 activity after carbachol treatment. Levels of α2 AMPK activity were only slightly above those observed for preimmune controls and the fold increase for α2-containing complexes was 1.5-fold. As activation by carbachol results in a 3-5 fold increase in AMPK activity, this suggests that carbachol mediates its downstream effects through AMPKα1-containing complexes.

**AMPK activation by carbachol is independent of cellular AMP:ATP.** The AMPK pathway is activated by cellular stress manifesting as perturbations in the AMP:ATP ratio or by alterations in intracellular Ca2+. The action of carbachol on muscarinic receptors increases intracellular calcium via G-protein coupled activation of phospholipase C (28). However, carbachol is reported to deplete cellular ATP concentrations in primary acinar cells by 15-20% through sodium pump stimulation (20). To determine which mechanism is required to activate AMPK in SH-SY5Y cells, intracellular nucleotide concentrations were measured in response to carbachol exposure. SH-SY5Y cells were treated with either hydrogen peroxide to activate AMPK by depletion of ATP, or carbachol. Increased AMPK activity was confirmed in response to both treatments (Table 1) and intracellular nucleotides levels assayed by ion exchange chromatography. As expected, AMP:ATP and ADP:ATP ratios were substantially increased after hydrogen peroxide treatment but there was no change in intracellular nucleotides levels after carbachol treatment (Table 1) suggesting that alterations in the AMP:ATP ratio do not mediate the carbachol-induced AMPK activation.

**Carbachol activation of AMPK requires muscarinic receptors, phospholipase C activation and increases in intracellular Ca^{2+}.** As well as muscarinic receptors, SH-SY5Y cells also express nicotinic acetylcholine receptors (nAChR; (29)) which can be activated by carbachol. In order to rule out a contribution from nAChRs, SH-SY5Y cells were treated with the muscarinic receptor-specific agonist, pilocarpine and antagonist, atropine. In response to 10µM pilocarpine, AMPK activity increased two-fold, whereas pre-treatment with 10µM atropine for 30 min completely prevented carbachol-induced AMPK activation (Fig. 2a). These data suggest that carbachol activates AMPK through a muscarinic, and not nicotinic, receptor-mediated pathway. SH-SY5Y cells predominantly express Gq protein-coupled M3 muscarinic receptors which, upon activation, initiate a downstream cascade that involves the stimulation of PLCβ and generation of IP3 (28). To confirm that AMPK can be activated via this pathway, we treated SH-SY5Y cells with the PLC activator m-3M3FBS, which resulted in a substantial increase in AMPK activity. Pre-treatment with a low concentration of U73122 (1µM), an inhibitor of PLC, diminished the carbachol activation of AMPK by over 50% (Fig. 2b). Finally, to determine whether carbachol activation of the muscarinic receptor results in an increase in intracellular calcium in SH-SY5Y cells we performed live cell imaging on cells loaded with the membrane-permeable fluorescent calcium indicator, Fluo-4-AM. Upon application of 10µM carbachol SH-SY5Y cells showed a transient elevation of intracellular calcium (Figure 2c, left panel). The rise in intracellular calcium was prevented by pre-treatment with 10µM atropine for 15 min prior to exposure to carbachol in presence of atropine (Fig. 2c, right panel). These data suggest that in SH-SY5Y cells, AMPK is activated by carbachol via the classical pathway, which requires the Gq-coupled muscarinic M3 receptor, PLC activity and increased intracellular Ca^{2+}.

**CaMKKβ mediates carbachol activation of AMPK.** As intracellular calcium concentrations increase substantially in SH-SY5Y cells after exposure to carbachol, CaMKKβ is therefore a likely candidate through which carbachol exerts its effects on AMPK. To determine its involvement, SH-SY5Y cells were treated with carbachol for 5...
minutes with or without a pre-treatment of STO609, a pharmacological inhibitor of CaMKKα and β (30). Activation of AMPK by carbachol was prevented by STO609 (Fig. 3a), suggesting a role for CaMKK. AMPK is also regulated by LKB1 phosphorylation, which is not reported to be sensitive to calcium flux. We determined that LKB1 is functionally active in SH-SY5Y cells by treatment with AICAR, a pharmacological activator of AMPK, which when metabolised, yields the AMP mimetic ZMP. We observed a small but significant 2-fold increase in AMPK activity suggesting that a functional LKB1-mediated pathway exists in SH-SY5Y cells (Fig. 3b) as CaMKKβ is not activated in response to AICAR treatment (6). As SH-SY5Y cells possess both upstream kinase pathways, they were transfected with siRNA against CaMKKβ and LKB1 to determine specifically which is involved in carbachol-mediated AMPK activity. Despite a number of attempts using different antibodies, we were not a ble to measure knockdown of endogenous LKB1 and CaMKKβ by western blotting due to lack of sensitivity. However, using more sensitive activity assays we determined that the activity of both kinases was reduced by at least 50% after siRNA treatment as measured by their ability to phosphorylate recombinant AMPK in vitro (Fig. 3c). This degree of knock-down is consistent with previous published studies using these siRNAs (8,25). In addition, we and others have found that SH-SY5Y cells have a low transfection efficiency, routinely less than 50% ((31,32), which may also contribute to the incomplete knock-down of protein expression. RNA inhibition of each upstream kinase reduced endogenous basal AMPK activity by almost 50% (Fig. 3d). However, inhibiting LKB1 activity did not diminish AMPK activation by carbachol (4.4-fold; Fig. 3d). In contrast, carbachol activation of AMPK was reduced from 3.5-fold in control cells to 1.8-fold in cells in which expression of CaMKKβ was down-regulated (Fig. 3d). Therefore CaMKKβ, not LKB1, is required for activation of AMPK by carbachol.

AMPK activation inhibits neuropeptide mRNA expression. Both AMPK and carbachol-activated muscarinic receptor pathways are implicated in the regulation of food intake. We therefore determined whether carbachol and other modulators of AMPK activity altered mRNA expression of neuropeptides that regulate appetite and energy balance. We began by characterising the SH-SY5Y system with respect to known direct and indirect activators of AMPK. Treatment with 10µM carbachol produced robust AMPK activation (Fig. 4a) and RNA was prepared from cells treated at the same time. Quantitative real time RT-PCR was performed using primers against neuropeptide Y (NPY), Melanin Concentrating Hormone (MCH), Agouti-related peptide (AgRP) and proopiomelanocortin (POMC). We were unable to detect POMC mRNA in SH-SY5Y cells although NPY, AgRP and MCH mRNA were readily amplified (an average of 21, 25 and 27 cycles of PCR respectively). In contrast with previously reported data (18), we detected no significant change in NPY mRNA in response to AMPK activation (Fig. 4b). Surprisingly, we found a substantial decrease in AgRP and MCH mRNA expression 4-6 hours after carbachol exposure (Fig. 4b). To confirm that AMPK itself was critical for the observed changes, we repeated the experiment using a newly described direct pharmacological activator of AMPK, A-769662 (33,34). Treating SH-SY5Y cells with A-769662 for 1 hour produced an AMPK activation of over 3-fold compared with control (Fig. 4a) and there was a significant decrease in both AgRP and MCH mRNA expression (Fig. 4b) although the inhibition wasn’t as pronounced as for carbachol. We attempted to abrogate the effect of carbachol on mRNA expression by pre-treatment of SH-SY5Y cells with STO609 or Compound C, a pharmacological inhibitor of AMPK. Pre-treating SH-SY5Y cells with Compound C prevented the activation of AMPK by carbachol with an effect similar to that of STO609 (Fig 4a). As before, there was no change observed in the expression of NPY after STO609 treatment (Fig. 4a). However, rather than returning to control values, treatments that inhibited endogenous AMPK activity caused a significant increase in the expression of AgRP and MCH mRNA (Fig. 4c) suggesting that basal AMPK activity exerts tight control over the regulation of neuropeptide expression.

DISCUSSION

The data presented in the current study show that the muscarinic receptor agonist carbachol causes activation of AMPK in SH-SY5Y cells via a
CaMKKβ signalling pathway. The activation may occur through the Gq protein-coupled M3 receptor predominant in SH-SY5Y cells (21), causing release of calcium from intracellular stores through activation of phospholipase C. Subsequently, this results in a repression of AgRP and MCH mRNA expression, neuropeptides involved in the propagation of signalling downstream of leptin in the hypothalamus. Inhibiting endogenous AMPK activity can reverse this effect, resulting in significant increases in AgRP and MCH levels. The substantial mRNA changes observed suggest that tight regulation of basal AMPK activity is critical in modulating the expression of these neuropeptides.

Our data contrasts with a previous study in which exposure to carbachol increased NPY gene expression in SH-SY5Y cells (35). This may be due to differences in experimental procedure as the carbachol treatment time was considerably longer (3h compared with 5min in this study). In addition, the authors normalised their northern blots with the housekeeping gene GAPDH, which has since been shown to exhibit variability of expression under various conditions (36,37). The normalisation carried out on the quantitative RT-PCR in this study was achieved using 18S expression, which did not vary during any of the SH-SY5Y treatments (data not shown).

Our results represent the first example of a CaMKKβ- rather than an LKB1-specific AMPK activation pathway in neuronal cells in which both upstream kinases are present. Indeed, although there have been very recent reports of CaMKKβ-specific pathways (38,39), there are few other published incidences of CaMKKβ-specific AMPK pathways in cells shown to express LKB1 as well. Thrombin activates AMPK via CaMKKβ in primary endothelial cells resulting in phosphorylation of the AMPK substrate, ACC (25). Inhibiting endogenous LKB1 does not prevent AMPK activation by thrombin, although LKB1 can phosphorylate AMPK in response to other stimuli in these cells. In T lymphocytes, AMPK can be activated by cellular stress inducing a high AMP:ATP ratio by a mechanism that is insensitive to STO-609, likely through LKB1 (40). However, triggering the T cell antigen receptor complex in these cells promotes an influx of Ca2+ and activates AMPK via a CaMKKβ-specific route. Interestingly, in cells in which a CaMKKβ-specific pathway has been identified, the predominant catalytic isoform is AMPKα1, and α2 is expressed at a low level if at all (Fig. 2) (25,40).

CaMKKα and β are highly expressed in brain (41). By contrast, LKB1 is not normally highly active in neuronal tissue and there is only one reported case of Peutz-Jeghers syndrome involving a neuronal tumour (42). Thus, a neuronal CaMKKβ-specific pathway may represent the typical mode of AMPK regulation in brain. However, in acute cerebral ischaemia, AMP levels are significantly increased (43) and LKB1 mRNA expression is upregulated after the generation of permanent focal ischaemia in a mouse model (44). Additionally, increased intracellular AMP potentiates the regulation of the GABAβ receptor by AMPK in the hippocampus (14). As CaMKKβ is insensitive to changes in AMP:ATP (7), it is likely that this effect is mediated by LKB1 and may mimic the physiological response that occurs during metabolic stress or ischaemia. These data and the current study reinforce a hypothesis in which both AMPK kinases are present in the same neuron rendering it capable of responding either to normal excitation or pathological stimuli (11,45).

Both AMPK and the muscarinic M3 receptor have been implicated in the regulation of food intake. M3 receptors are highly expressed in both arcuate and lateral hypothalamic areas, governing first (AgRP) and second order (MCH) neuronal responses respectively (22). M3 knockout mice are hypophagic and lean and AgRP mRNA expression is significantly increased compared with control, as might be expected with decreased food intake. The authors suggest that the function of M3 is therefore downstream of first order neurons as they observe a slight decrease in MCH mRNA levels (22). However, our data may explain the observed increase in AgRP as knocking out the M3 receptor would decrease AMPK activity and promote AgRP expression. Our data is derived from a cell line in which a known subset of muscarinic receptors is expressed (21), which has the advantage of allowing the study of complex pathways to be simplified. However, a drawback of this system is that projections from other neuronal areas cannot be taken into account. This becomes very relevant in the hypothalamus in...
which regions such as the arcuate nucleus possess different subpopulations of neurons with distinct roles in the regulation of feeding (46). Future work will require a more \textit{ex vivo} approach in which a detailed dissection of the region can be undertaken.

In summary, our data suggest that neuronal LKB1-AMPK or CaMKKβ-AMPK pathways may exist that are capable of selective responses depending on neuronal receptor type, upstream kinase and AMPK isoform activation.

REFERENCES

FOOTNOTES
This work was supported by the Medical Research Council (D.C.) and a Wellcome Trust / Imperial College Value in People Award (C.T.).

The abbreviations used are: AICAR, 5-aminoimidazole-4-carboxamide ribonucleoside; AgRP, Agouti-related peptide; AMPK, AMP-activated protein kinase; MCH, Melanin Concentrating Hormone; NPY, neuropeptide Y; POMC, proopiomelanocortin.
FIGURE LEGENDS

Fig 1. Carbachol rapidly activates AMPK. SH-SY5Y cells were treated with different concentrations of carbachol for 5 min (a) or with 10µM carbachol for varying times (b). AMPK was immunoprecipitated from whole cell lysates (100µg) using a rabbit pan-β antibody and activity analysed by AMARA peptide substrate assay. AMPK activity is shown as nmol/min/IP (n = 4 ± SEM; ** p< 0.01). (c) Representative blot (n=3) of lysates (50µg) resolved by SDS-PAGE and subjected to western blotting using phospho-acetyl CoA carboxylase (pACC; top panel) or actin (bottom panel) antibodies. (d) Untreated (con) or carbachol-treated (10µM, 5 min; carb) SH-SY5Y cells were harvested and cell lysates (100µg) used for 3 rounds of immunoprecipitation of endogenous AMPK with α1- or α2-specific antibodies. Mock immunoprecipitations were carried out using rabbit pre-immune serum. AMPK activity was measured using the AMARA peptide substrate assay (n = 3 ± SD; ** p< 0.01)

Fig 2. AMPK is activated via the classical muscarinic M3 receptor pathway. (a) SH-SY5Y cells were treated with pilocarpine (10µM, 30 min), carbachol (10µM, 5 min), atropine (10µM, 20 min) or pre-treated with atropine (10µM, 20 min) followed by a subsequent exposure to carbachol (10µM, 5 min). (b) SH-SY5Y cells were treated with carbachol (10µM, 5 min), m-3M3FBS (100µM, 2 min) or pre-treated with U73122 (1µM, 30 min) followed by a subsequent exposure to carbachol (10µM, 5 min). (a, b) In each case, cells were rapidly lysed and endogenous AMPK immunoprecipitated and assayed from whole cell lysates (100µg) as before (n = 3 ± SD; *p>0.05; ** p<0.01). (c) SH-SY5Y cells plated on coverslips were loaded with the calcium-sensitive dye, Fluo 4 AM and either exposed to carbachol (10µM; left panel) or pretreated with atropine (10µM, 15 min) before carbachol exposure in the presence of atropine (right panel). Maximal fluorescence was obtained by treatment with 10µM ionomycin and the signal expressed as the ratio of F/Fmax. In each case, representative traces are shown and similar results were obtained from at least 3 independent experiments.

Fig 3. Inhibiting CaMKKβ prevents AMPK activation by carbachol. (a) SH-SY5Y cells were treated with carbachol (10µM) either with or without pre-treatment with STO609 (10µg/ml, 1h). AMPK activity was assayed as before and activities are shown ± SD (n = 4, *p<0.05, **p <0.01) (b) SH-SY5Y cells were treated with AICAR (2mM) and AMPK assayed as before (n = 3 ± SD) (c) CaMKKβ and LKB1 were immunoprecipitated from untreated or siRNA treated SH-SY5Y lysates (200µg) and used to reactivate recombinant bacterially expressed AMPK (rAMPK). AMPK activity was then assayed and activities are shown as percentage of control ± SD (d) SH-SY5Y cells were transfected with siRNA against CaMKKβ or LKB1 and treated 48hr later with 10µM carbachol for 5 min. AMPK activity was assayed as before (n = 4 ± SD, ** p<0.01).

Fig 4. AMPK activity modulates orexigenic neuropeptide mRNA expression. SH-SY5Y cells were treated with AMPK activators (Carbachol, 10µM; A769662, 200µM) or inhibitors (STO609, 10µg/ml; Compound C, 10µM). (a) AMPK was immunoprecipitated from untreated control or treated SH-SY5Y cell lysates and assayed as before (n = 3, *p<0.05, **p <0.01). (b)+(c). Total RNA from untreated control or treated SH-SY5Y cell lysates (10ng) was subjected to quantitative RT-PCR using primers for NPY, AgRP and MCH and expression normalised using 18S. (n = 4 ± SD; *p<0.05; ** p<0.01).
Table 1. Intracellular nucleotide ratios after treatment with carbachol or hydrogen peroxide (n = 3 ± SD).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>AMPK activity (nmol/min/IP)</th>
<th>AMP:ATP</th>
<th>ADP:ATP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.0069 ± 0.0014</td>
<td>0.021 ± 0.0007</td>
<td>0.046 ± 0.006</td>
</tr>
<tr>
<td>Carbachol (10μM)</td>
<td>0.0209 ± 0.0001</td>
<td>0.021 ± 0.0006</td>
<td>0.049 ± 0.003</td>
</tr>
<tr>
<td>Hydrogen Peroxide (500μM)</td>
<td>0.0352 ± 0.0003</td>
<td>0.235 ± 0.0059</td>
<td>0.243 ± 0.049</td>
</tr>
</tbody>
</table>
Figure 1

(a) AMPK Activity (nmol/min/IP) vs. Carbachol treatment (min)

(b) AMPK Activity (nmol/min/IP) vs. Carbachol treatment (min)

(c) Western blot analysis of p-ACC and Actin levels across different Carbachol treatment times.

(d) AMPK Activity (nmol/min/IP) for different rounds of α1 and α2 IPS, Con, and Preimmune controls.
Figure 2

(a) AMPK Activity (nmol/min/IP)

- Control
- Pilocarpine
- Carbachol
- Atropine
- Atropine + Carbachol

(b) AMPK Activity (nmol/min/IP)

- Control
- Carbachol
- m-3M3FBS
- U73122 + Carbachol

(c) F/Fmax vs Time (s)

- Carb
- Iono
- Atropine
Figure 3

a

AMPK activity (nmol/min/IP)

Control Carbachol STO609 + Carbachol

b

AMPK activity (nmol/min/IP)

Control AICAR

LKB1 RNAi CaMKKβ RNAi

c

AMPK activity (%)

LKB1 RNAi CaMKKβ RNAi

3.5-fold 4.4-fold 1.8-fold

Carbachol (10μM) Control + LKB1 RNAi + CaMKKβ RNAi
Figure 4

a

AMPK activity (Fold change)

Con Carbachol A-769662 STO609 STO609 + Carbachol Compound C + Carbachol Vehicle

b

Normalised mRNA expression

Con 2h 4h 6h 8h

NPY AgRP MCH AgRP MCH Carbachol A-769662

STO609 and Carbachol Compound C and Carbachol
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